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Senescent cell antigen is immunologically related to band 3

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ABSTRACT **IgG autoantibodies in human serum selectively bind to a glycopeptide antigen that appears on senescent and damaged cells *in situ*. We identified the membrane protein from which the senescent cell antigen is derived by using a phagocytosis-inhibition assay and immunoautoradiographic gel staining and electroblotting techniques. Results of the phagocytosis-inhibition assay revealed that only the purified transmembrane glycoprotein designated "band 3" and senescent cell antigen inhibited the phagocytosis of erythrocytes induced by IgG eluted from senescent erythrocytes. Purified spectrin, syndein, band 4.1, actin, glycophorin A, and intact or desialylated sialoglycoprotein periodic acid/Schiff (PAS) staining bands 1–4 containing glycophorins A, B, and C did not inhibit phagocytosis. Specific antibodies against the senescent cell antigen and erythrocyte band 3 were used to identify the membrane protein from which the senescent cell antigen is derived. Band 3-related polypeptides (M_rs ≈ 60,000, 42,000, and 18–26,000) were identified in erythrocyte ghosts prepared in the presence of diisopropyl fluorophosphate, phenylmethylsulfonyl fluoride, and EDTA by immunoautoradiography with anti-band 3. Antibodies to senescent cell antigen reacted with band 3 and the same lower M_r band 3-related polypeptides. Thus, the senescent cell antigen is immunologically related to band 3.**

Approximately 360 billion human erythrocytes (RBCs) are removed each day from the circulation. A specific recognition system has developed that permits the removal of senescent and damaged cells and spares intact mature cells (1–4). An antigen (M_r = 62,000) that appears on the surface of senescent or damaged cells (3) is recognized and bound by the antigen-binding Fab region (2) of an IgG autoantibody in serum (1). The Fc region of the autoantibody is then recognized and bound by a macrophage, which proceeds to phagocytize the cells. It appears that senescent, stored, or damaged nucleated cells may be removed by a similar mechanism because the senescent cell antigen can be detected on these cells (2, 3).

Previous experiments with senescent cell antigen, purified by affinity chromatography with IgG eluted from senescent cells, indicated that the antigen is a M_r ≈62,000 protein migrating in the band 4.5 region of $NaDodSO_4$/polyacrylamide gradient gels (3). Because the senescent cell antigen can be isolated from pure sialoglycoprotein mixtures prepared by the method of Marchesi and Andrews (5) and stains faintly with dansylhydrazine, it appears to be glycosylated (3). The sialoglycoprotein mixtures contain periodic acid/Schiff (PAS) staining bands 1–4 (6) (Steck's nomenclature; ref. 7) but do not contain the transmembrane glycoprotein designated "band 3" (8, 9) containing ≈7% carbohydrate (7). Only one band could be isolated with the senescent cell IgG column (3) even after repeated passages of sialoglycoprotein mixtures through it, a procedure that depleted the mixtures of the M_r ≈62,000 peptide and should have facilitated binding of a "parent" molecule if one existed (10). Failure to detect another molecule by this procedure suggested that either the senescent cell antigen was not derived from another molecule or that the "parent" molecule did not copurify with the sialoglycoproteins (10). Because mature RBCs cannot synthesize protein, it is probable that the senescent cell antigen, which appears to be a terminal differentiation antigen, was generated by modification of a preexisting protein, resulting in a change in configuration to that of an antigenically "new" molecule.

We have postulated that band 3 might be the progenitor of the M_r ≈62,000 glycopeptide because it contains relatively small amounts of carbohydrate and does not copurify with sialoglycoprotein PAS bands 1–4 (10). Results of the experiments presented here demonstrate that the senescent cell antigen is antigenically related to band 3, an integral membrane protein that is involved in anion transport across the RBC membrane (11, 12) and appears to be the binding site for glyceraldehyde-3-phosphate dehydrogenase (13), aldolase (14), phosphofructokinase (15), hemoglobin (16), and syndein (17).

MATERIALS AND METHODS

Cell Separation and Storage. RBCs were separated into young, middle-aged, and old populations in Percoll gradients as described (4). Platelets and white cells formed bands at the top of the gradient and were removed (4). Young RBCs were in the least dense fraction (ρ = 1.090) and old RBCs were found in the most dense fraction (ρ = 1.120) as determined by ^{59}Fe labeling *in situ* (4). Young RBCs were stored in Alsever's solution at 4°C for 1–2 weeks.

Isolation of IgG from Senescent RBCs. IgG was isolated as described (2, 3). Briefly, middle-aged and old RBCs were washed three times with 50–100 vol of Dulbecco's phosphate-buffered saline, pH 7.4. RBC membranes were prepared by digitonin lysis and washed three times with phosphate-buffered saline, and IgG was eluted with 0.1 M glycine·HCl buffer, pH 2.3. Eluates were neutralized with 1 M NaOH and concentrated by using an Amicon Diaflow with a PM 10 filter. IgG was isolated from eluates by affinity chromatography with protein A-Sepharose 4B (3).

Isolation of the Senescent Cell Antigen. IgG eluted from senescent RBCs and purified by chromatography on protein A-Sepharose (Pharmacia) was coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia). These senescent cell IgG columns were used to isolate the senescent cell antigen from sialoglycoprotein preparations as described (3, 10) or from RBC

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Abbreviations: PAS, periodic acid/Schiff (stain for sialoglycoproteins); RBCs, erythrocytes.

ghosts from which peripheral membrane proteins were removed with 10 vol of ice-cold 0.1 M NaOH (9). The membranes stripped of peripheral proteins were dissolved in 10 mM Tris·HCl, pH 8.5/0.5% Triton X-100/0.03% $LiDodSO_4$ and centrifuged at 30,000 × *g* for 1 hr at 4°C.

Sialoglycoprotein Preparations. Right-side-out RBC ghosts were suspended in a solution of 0.3 M lithium diiodosalicylate and partitioned in a phenol/water mixture according to the procedure of Marchesi and Andrews (5). These preparations contain PAS staining bands 1–4 (3, 6) and include glycophorins A, B, and C but not band 3 (6). The senescent cell antigen was removed from sialoglycoprotein mixtures by passage through the senescent cell IgG column. Glycophorin A was purified by the method of Furthmayr (6). Sialoglycoprotein mixtures were separated on a Sepharose 6B column equilibrated with 5 mM phosphate buffer, pH 8/25 mM NaCl/0.1% Ammonyx-LO (Onyx Chemical, Jersey City, NJ). The first peak contained pure glycophorin A (6). For some experiments, the glycophorin A separated by column chromatography was labeled with dansyl chloride and further purified by preparative polyacrylamide gel electrophoresis (6).

Desialylation of Glycoproteins. Purified glycophorin A and the sialoglycoprotein mixtures were desialylated by using both a chemical method (dilute acid) and an enzymatic method (neuraminidase) as described by Prohaska *et al.* (18). The chemical method results in 100% desialylation of isolated glycoproteins, whereas the enzymatic method results in 90% desialylation (18).

Purification of Peripheral Membrane Proteins. Spectrin heterodimers were purified as described by Bennett and Branton (19) but modified so that rate zonal sedimentation was performed by the method of Goodman and Weidner (20). RBC actin was prepared by the method of Nakashima and Beutler (21). Band 2.1 (syndein) and protein 4.1 were isolated essentially by the method of Tyler *et al.* (22) with the modifications of Goodman *et al.* (23).

Band 3 Purification. White cells were removed from RBCs by using cellulose columns as described by Beutler *et al.* (24) or by density centrifugation on Percoll gradients (4). Band 3 was isolated both from intact RBCs and from trypsin-treated RBCs because mild trypsinization of whole RBCs cleaves glycophorin A and C but spares band 3 (6, 25). For trypsinization, washed RBCs were incubated with phosphate-buffered saline containing 1 mM ATP overnight at 24°C with 100 μg of trypsin per ml (25). Digestion was terminated by the addition of 0.5 mM diisopropyl fluorophosphate. RBCs were washed three times in phosphate-buffered saline (pH 7.4) and lysed in 5 mM sodium phosphate buffer (pH 8.0). Diisopropyl fluorophosphate (0.5–1.0 mM), phenylmethylsulfonyl fluoride (100 μg/ml), and EDTA (1 mM) were used throughout all procedures to avoid artifactual proteolysis. RBC ghosts were stripped of peripheral membrane proteins by the addition of 10 vol of 0.1 M NaOH at ice temperature. Band 3 was purified by gel-filtration chromatography (8, 9) or ion-exchange chromatography (26). For gel-filtration chromatography, ghosts were dissolved in 2% $LiDodSO_4$/5 mM dithiothreitol/0.02% NaN_3 and chromatographed on a Sepharose-4B column (5 × 18 cm) equilibrated with 10 mM Tris·HCl, pH 8.5/0.25% $LiDodSO_4$. Isolation of band 3 by ion-exchange chromatography on Affi-Gel 102 (Bio-Rad) was performed as described by England *et al.* (26) with the following modifications. The pellet derived from 0.1 M NaOH-treated ghosts was dissolved in 80 ml of 50 mM Tris acetate/0.5% Triton X-100/0.03% $LiDodSO_4$/0.02% NaN_3/0.5 mM diisopropyl fluophosphate, pH 8.0. The solution was centrifuged at 30,000 × *g* for 1 hr. The supernatant was loaded at 25 ml/hr onto an Affi-Gel 102 column equilibrated with the same buffer. The column was washed with 200 ml of equilibration buffer and developed with a stepped gradient of 0–250 mM NaCl in 25 mM Tris acetate/1.5 mM sodium phosphate buffer/0.5% Ammonyx LO/0.03% $LiDodSO_4$/0.02% NaN_3/0.25 mM diisopropyl fluorophosphate/1 mM EDTA, pH 8.1. Peaks were located by monitoring ultraviolet absorbance and were dialyzed against 10 mM imidazole·HCl/0.5 mM diisopropyl fluorophosphate, pH 8.0. As described by England *et al.* (26), band 7 and zone 4.5 were not bound to the column. The PAS staining profile was eluted with 25–50 mM NaCl. Band 3 was eluted between 100 and 150 mM NaCl.

$NaDodSO_4$/Polyacrylamide Gel Electrophoresis. Proteins were analyzed on two different gel systems: (*i*) 4–30% polyacrylamide gradient gels containing $NaDodSO_4$, with a continuous buffer system (3, 27); and (*ii*) 7% polyacrylamide gels containing $NaDodSO_4$, with the discontinuous buffer system of Laemmli (28). A more complete description of the electrophoresis conditions has been described (3, 29). The sample buffer was 10 mM Tris·HCl, pH 6.8/1 mM EDTA/40 mM dithiothreitol/5% glycerol/2% $NaDodSO_4$/0.2 mg of Pyronin Y per ml. Gels were stained for protein with Coomassie blue and for glycoprotein with dansylhydrazine.

Erythrophagocytosis-Inhibition Assay. IgG eluted from senescent RBCs (7.5 μg) was absorbed with a 10-fold molar excess of each membrane component, except the senescent cell antigen, for 2 hr at 24°C. The quantity of senescent cell antigen used for absorption was equal to the amount isolated from 250 ml of whole blood. The absorbed IgG was dialyzed against phosphate-buffered saline and then incubated with stored RBCs because these cells have the senescent cell antigen on their surface (1–3). RBCs were washed and incubated with autologous macrophage cultures (1–4). The phagocytosis-inhibition assay was performed as described (1, 4).

Rabbit Antisera to Membrane Proteins. Antisera to the senescent cell antigen were prepared by injecting rabbits with the senescent cell antigen isolated from both lymphocytes and from RBCs by affinity chromatography (3). The reactivity of antisera to the senescent cell antigen was the same regardless of the cell type used for isolation, as determined by immunoautoradiography. The senescent cell antigen has been demonstrated on nucleated cells, including lymphocytes (3). Rabbits received two injections 1 week apart, followed 1 month later by two more injections. Rabbits were injected at monthly intervals thereafter and bled weekly after the fifth injection. Antigens were emulsified in complete Freund's adjuvant for the first injection and in incomplete Freund's adjuvant for all other injections. Antisera to band 3 were prepared by injecting rabbits once a month with purified band 3 polypeptide.

Immunostaining of Membrane Proteins. Immunoautoradiography was performed by the gel overlay method of Granger and Lazarides (30) with the minor modifications described in the text or by the electroblot technique (31) with the modifications described below. Membrane proteins were separated on 4–30% polyacrylamide gradient gels and transferred electrophoretically to nitrocellulose sheets (31). Nitrocellulose sheets were treated with 0.1% glutaraldehyde to prevent diffusion of proteins during processing of blots. At this concentration, glutaraldehyde causes covalent binding of protein amino groups to each other but does not alter antigenicity or antibody binding (32, 33). Free binding sites on the nitrocellulose sheets were blocked by incubation with 10% ethanolamine (pH 8.0) for 1–3 hr at 37°C, followed by an incubation with 3% bovine serum albumin/0.9% NaCl for 1 hr at 24°C. Blots were incubated with rabbit antisera to membrane proteins overnight at 24°C. After washing with 0.9% NaCl/0.05% Tween 20, blots were incubated with ^{125}I-labeled protein A (^{125}I-protein A; New England Nuclear, 70–100 μCi/μg; 1 Ci = 3.7 × 10^{10} Bq) in 10 ml of

3% bovine serum albumin for 2 hr at 24°C (3 μCi of ^{125}I-protein A were utilized per blot). Blots were washed, dried, and exposed to Kodak X-Omat RP film at −40°C for 12–72 hr in a Kodak cassette with intensifying screen. Transfer of polypeptides was monitored by loss of Coomassie blue-staining bands from the gel and by the appearance of Amido black-staining bands on the nitrocellulose paper. Transfer of polypeptides was 70–90% efficient.

RESULTS

Absorption of the Phagocytosis-Inducing Ability of IgG Eluted from Senescent RBCs by Band 3 and Senescent Cell Antigen. In order to test the hypothesis that the senescent cell antigen was derived from band 3 (10), an erythrophagocytosis inhibition assay was performed (2, 3). IgG (10 μg) eluted from senescent RBCs was incubated for 2 hr at 24°C with purified spectrin, band 2.1, band 3 (Fig. 1), glycophorin A, sialoglycoprotein mixtures containing PAS staining bands 1–4, desialylated sialoglycoprotein mixtures, band 4.1, senescent cell antigen, or actin. The absorbed IgG was then incubated with stored RBCs, which served as target cells for the phagocytosis assay because they have accessible senescent cell antigen (1, 2, 10). The RBCs were washed and incubated with autologous macrophage cultures. Absorption of IgG isolated from senescent RBCs with band 3 or with the purified senescent cell antigen abolished its ability to induce phagocytosis of stored RBCs indicating that no free antigen-binding IgG remained in solution (Table 1). Absorption of IgG eluted from senescent RBCs with spectrin, syndein, glycophorin A, sialoglycoprotein mixtures containing PAS staining bands 1–4, desialylated glycoprotein mixtures, band 4.1, and actin did not alter its phagocytosis-inducing ability, indicating the presence of free antigen-binding IgG (Table 1). These results indicate that band 3 and the M_r 62,000 glycopeptide carry the antigen determinants recognized by IgG eluted from senescent cells and suggest that the senescent cell antigen is derived from band 3. The results confirm our previous report that the senescent cell antigen is not a desialylated glycophorin based on gel transfer data and a phagocytosis-inhibition assay (34, 35). Moreover, band 3 isolated from RBCs after mild trypsinization still absorbed the phagocytosis-inducing ability of senescent cell IgG (% phagocytosis, 2 ± 2). Trypsinization of whole RBCs digests glycophorins A and C (6) but spares band 3 (7).

Band 3-Related Polypeptides in RBC Membranes. Antibodies to band 3 were used to detect band 3-related polypeptides in human erythrocyte membranes by using an immunoautoradiographic gel staining technique (30). Human RBC membrane proteins were separated by using NaDodSO$_4$/7% polyacrylamide gel electrophoresis with the discontinuous buffer system of Laemmli (28). Erythrocyte membrane proteins of M_r > 35,000 are resolved with this system. The gel was fixed, equilibrated with a physiological ionic strength buffer, incubated with anti-band 3 IgG, and then stained with ^{125}I-protein A. Comparison of the Coomassie blue-stained gel with the corresponding immunoautoradiogram (Fig. 2*A*) shows that the anti-band 3 IgG stains band 3 and additional polypeptides at $M_r \approx$ 64,000 (faint staining was reproducibly observed), 60,000, and 42,000 and at the position of the tracking dye (M_r < 34,000).

When erythrocyte membrane proteins were separated on 4–30% polyacrylamide gradient gels (3, 27) and transferred to nitrocellulose paper, followed by overlay with anti-band 3 and then ^{125}I-protein A, a more complete staining pattern was observed (Fig. 2*B*). Distinct staining of band 3, bands at $M_r \approx$ 64,000, 60,000, and 42,000, and at least four bands at $M_r \approx$ 18–26,000 was observed. In addition, faint staining at M_rs ≈ 80,000, 58,000, 54,000, 37,000, and 32,000 was routinely observed. Absorption of anti-band 3 with the supernatant of NaOH-treated ghosts containing all of the peripheral membrane proteins and with PAS 1–4 conjugated to cyanogen bromide-activated Sepharose 4B did not result in decreased staining of any bands when

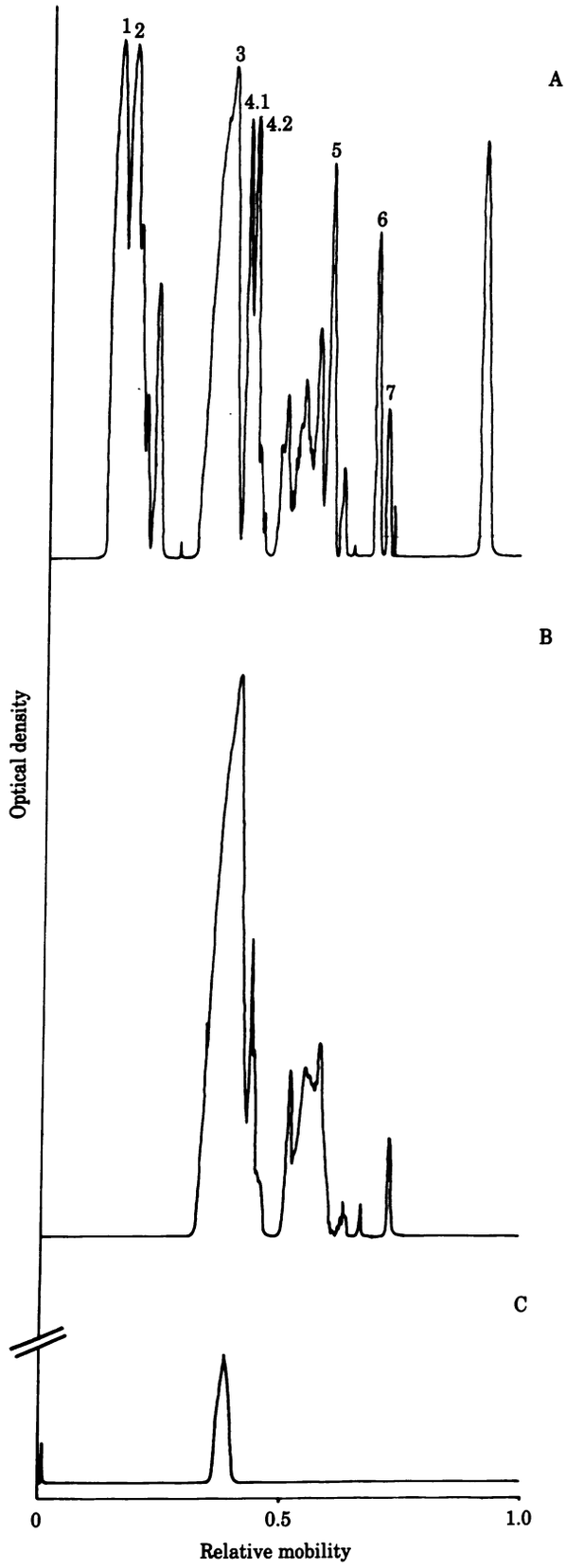

FIG. 1. Purification of band 3. (*A*) Human RBC ghost proteins. (*B*) RBC ghosts after treatment with 0.1 M NaOH. (*C*) Purified band 3.

Table 1. Phagocytosis-inducing ability of IgG eluted from senescent cells after absorption with RBC membrane components

Component used for absorption	Phagocytosis, % ± SD
None	30 ± 11
Spectrin	25 ± 6
Syndein	29 ± 7
Band 3	2 ± 3
Glycophorin A	34 ± 6
Sialoglycoprotein mixture	25 ± 7
Desialylated sialoglycoprotein MX (chemical)	37 ± 6
Desialylated sialoglycoprotein MX (enzymatic)	30 ± 11
Band 4.1	28 ± 14
Senescent cell antigen	0
Actin	27 ± 4

analyzed by immunoautoradiography (Fig. 2*B*). Thus, the antiserum to band 3 did not contain antibodies to peripheral membrane proteins or to the sialoglycoproteins PAS 1–4, further indicating that the band 3 preparation was homogenous. The presence of RBC membrane proteins that have M_rs less than that of band 3 and that stain with anti-band 3 antiserum in ghosts prepared with diisopropyl fluorophosphate, phenylmethylsulfonyl fluoride, and EDTA suggests that these polypeptides may be present in the intact RBCs.

Antibodies to the Senescent Cell Antigen Bind to Band 3. Antibodies to the senescent cell antigen were used to detect the polypeptide from which the antigen was derived. Membrane proteins were transferred from $NaDodSO_4$/4–30% polyacrylamide gels to nitrocellulose paper, followed by antiserum overlay. ^{125}I-protein A staining revealed binding of anti-senescent cell antigen to band 3 and to the band 3-related polypeptides described above (Fig. 3). No other membrane proteins were labeled, indicating that only band 3 and its related polypeptides carry the antigenic determinants of the senescent cell antigen.

DISCUSSION

Antibody to band 3 reacts with band 3 and three main polypeptide groups ($M_r \approx$ 60,000, 42,000, and 18–26,000). These polypeptides are present in RBC ghosts prepared with both diisopropyl fluorophosphate and EDTA to avoid artifactual proteolysis. Therefore, these immunoreactive lower M_r forms of band 3 are probably present *in vivo* and may represent physiologically significant proteolytic products of the parent molecule. These results are in agreement with our peptide mapping analysis, which demonstrates that band 3, the M_r 64,000 polypeptide, and the M_r 60,000 polypeptide are sequence-related proteins (unpublished data).

The phagocytosis-inducing ability of IgG eluted from senescent RBCs is abolished by absorption with purified band 3 but not by absorption with spectrin, bands 2.1, 4.1, actin, glycophorin A, or intact or desialylated PAS staining bands 1–4. In addition, IgG eluted from senescent RBCs and rabbit antibodies prepared against the senescent cell antigen isolated from white blood cells react with band 3. Recently we have identified immunoreactive analogues of RBC band 3 in white blood cells. This data will be presented elsewhere. Thus, the senescent cell antigen appears to be immunologically related to band 3 and may be derived from it.

Because band 3 is present at a concentration of $\approx 1 \times 10^6$ copies per RBC (7), whereas the senescent cell antigen is present at a concentration of $\approx$100 copies per cell aged *in situ* (1), it seems that a selective alteration of band 3 is required to produce the

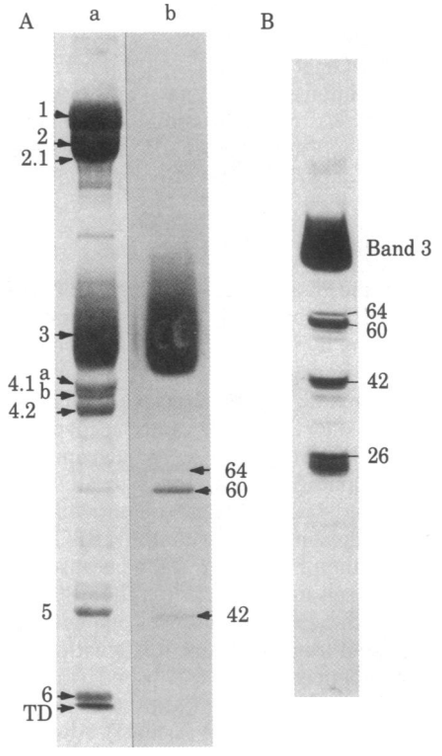

FIG. 2. (*A*) Detection of band 3-related polypeptides in RBC membranes with anti-band 3 IgG. Lanes: a, Coomassie blue-stained gel of RBC ghost proteins (64 μg of protein); b, immunoautoradiography with anti-band 3 (M_rs shown $\times 10^{-3}$). RBC membrane proteins were separated by electrophoresis on a $NaDodSO_4$/polyacrylamide (7%) slab gel with the discontinuous system of Laemmli (28). All subsequent incubations were performed with 250-ml volumes contained in a baking dish. The gel was fixed in 50% ethanol/10% acetic acid (6 hr at 22°C) and then neutralized in three changes of 50 mM Tris·HCl/100 mM NaCl/5 mM NaN_3, pH 7.5, for 24 hr. The gel was washed in buffer 1 (10 mM Tris chloride/140 mM NaCl/5 mM NaN_3/0.1 mM EDTA/0.1% gelatin, pH 7.5) for 24 hr, followed by a 24-hr incubation in buffer 1 to which anti-band 3 IgG (9 mg/ml) was added (1:1,000 dilution with buffer 1). Unbound IgG was washed away by washing for 3 days with buffer 1 (three changes) at 22°C. The gel was incubated in buffer 1 containing 2.5 μCi of ^{125}I-protein A (New England Nuclear) for 24 hr at 22°C. Unbound ^{125}I-protein A was removed by washing for 2 days with buffer 1, followed by washing for 1 day with buffer 1 without gelatin. The gel was stained in 0.1% Coomassie blue/47.5% ethanol/10% acetic acid and destained in 12.5% ethanol/5% acetic acid. The gel was dried and autoradiographed for 5 days (−70°C with intensifying screen). TD, tracking dye.

(*B*) Detection of band 3-related polypeptides in RBC membranes with anti-band 3 antiserum absorbed with the supernatant of NaOH-treated ghosts and PAS 1–4. RBC ghosts were treated with 0.1 M NaOH at ice temperature and centrifuged at 16,000 rpm (SS34) for 30 min. The supernatant containing peripheral membrane proteins was neutralized with 0.1 M HCl, concentrated by Amicon PM 10 ultrafiltration, and conjugated to cyanogen bromide-activated Sepharose 6MB (Pharmacia). Sialoglycoproteins (PAS staining bands 1–4) were prepared and conjugated to Sepharose 6B. Anti-band 3 antiserum was absorbed with each immunoabsorbant at 4°C for 48 hr. RBC ghost proteins were transferred from polyacrylamide gradient gels to nitrocellulose paper. The paper was overlaid with the absorbed antibody and then with ^{125}I-protein A. M_rs are shown $\times 10^{-3}$.

senescent cell antigen or that a subpopulation of band 3 molecules may be involved, or both. Alteration of membrane proteins by endogenous proteases, oxidation, oxidative cross-linking followed by proteolysis, addition or deletion of glycosyl groups, or peroxidation of adjacent lipids are possible mechanisms that require investigation (7, 36, 37).

Specific membrane changes during cellular aging can now be

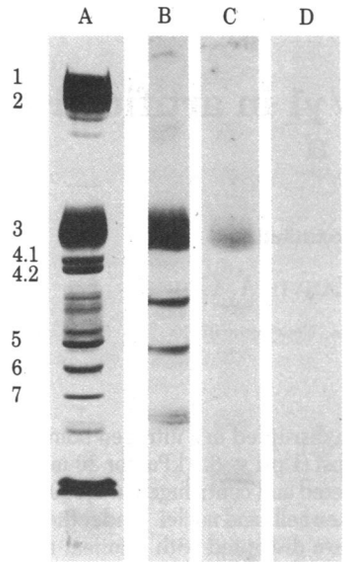

FIG. 3. Binding of the antibody against senescent cell antigen to band 3 and band 3-related polypeptides of RBC ghosts. Lanes: A, Coomassie blue stain of RBC ghost protein; B, immunoautoradiography with anti-band 3; C, immunoautoradiography with anti-senescent cell antigen; D, immunoautoradiography with nonimmune serum. Membrane proteins were separated on $NaDodSO_4$/polyacrylamide gradient gels. Polypeptides were visualized by Coomassie blue staining. Polypeptides to which antibody against band 3 or against senescent cell antigen binds were identified by electrophoretic transfer from polyacrylamide gels to nitrocellulose paper, followed by overlay of the nitrocellulose paper with antiserum and then with ^{125}I-protein A.

investigated by using both band 3 and the senescent cell antigen as probes. Dissection of changes occurring within band 3 during senescence may reveal mechanisms responsible for aging at a molecular and cellular level.

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